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# Tomato *E8* Encodes a C-27 Hydroxylase in Metabolic Detoxification of α-Tomatine during Fruit Ripening

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**Tomato (*Solanum lycopersicum*) contains α-tomatine, a steroidal glycoalkaloid that contributes to the plant defense against pathogens and herbivores through its bitter taste and toxicity. It accumulates at high levels in all the plant tissues, especially in leaves and immature green fruits, whereas it decreases during fruit ripening through metabolic conversion to the nontoxic esculeoside A, which accumulates in the mature red fruit. This study aimed to identify the gene encoding a C-27 hydroxylase that is a key enzyme in the metabolic conversion of α-tomatine to esculeoside A. The *E8* gene, encoding a 2-oxoglutalate-dependent dioxygenase, is well known as an inducible gene in response to ethylene during fruit ripening. The recombinant E8 was found to catalyze the C-27 hydroxylation of lycoperoside C to produce prosapogenin A and is designated as Sl27DOX. The ripe fruit of *E8/Sl27DOX*-silenced transgenic tomato plants accumulated lycoperoside C and exhibited decreased esculeoside A levels compared with the wild-type (WT) plants. Furthermore, *E8/Sl27DOX* deletion in tomato accessions resulted in higher lycoperoside C levels in ripe fruits than in WT plants. Thus, E8/Sl27DOX functions as a C-27 hydroxylase of lycoperoside C in the metabolic detoxification of α-tomatine during tomato fruit ripening, and the efficient detoxification by E8/27DOX may provide an advantage in the domestication of cultivated tomatoes.**

**Keywords:** 2-Oxoglutarate-dependent dioxygenase • a-Tomatine • Fruit ripening • E8 • Sl27DOX • Steroidal glycoalkaloid • Tomato *(Solanum lycopersicum)*

## Introduction

Tomato (*Solanum lycopersicum*) is the most produced vegetable globally and is also the most important commercial crop cultivated for both fresh markets and processed food products. Moreover, it is a well-known model species for plant science studies, especially for fruit-development research (Klee and Giovannoni 2011). After fertilization and fruit set, the development of tomato proceeds through fruit growth and ripening (Quinet et al. 2019). Significant changes in color, flavor, taste and texture of the fruit occur during the ripening process (Karlova et al. 2014).

Tomato is a climacteric fruit that exhibits a rapid increase in respiration and ethylene—a plant hormone—production at the onset of ripening. Ethylene plays an important role in fruit ripening, and its biosynthesis and response are highly transcriptionally regulated (Liu et al. 2015). The *E8* gene is one of the four genes transcriptionally induced by ethylene and ripening, and its expression is facilitated by exogenous ethylene treatment in a dose-dependent manner (Lincoln et al. 1987, Lincoln and Fischer 1988). Among several tomato ripening–associated proteins, RIN (RIPENING INHIBITOR), which belongs to a MADS-box transcription factor, functions as the primary ripening regulator and is required for complete ripening (Gapper et al. 2013, Ito et al. 2017). RIN directly regulates the expression of many tomato genes and binds to several gene promoters, including *E8* (Fujisawa et al. 2011, 2013, Qin et al. 2012). The *E8* promoter is a well-known tomato fruit ripening–specific promoter and is biotechnologically utilized to promote the expression of recombinant proteins in the fruit (He et al. 2008, Hirai et al. 2011). It belongs to the 2-oxoglutarate-dependent dioxygenase (DOX) superfamily (Kawai et al. 2014); however, the enzymatic activity of E8 remains unclear.

Significant alterations in both primary and plant-specialized metabolites, such as steroidal glycoalkaloids (SGAs), occur during tomato fruit development (Tohge and Fernie 2015). Approximately 100 tomato fruit SGAs have been annotated by metabolomic approaches (Iijima et al. 2008, Itkin et al. 2011, Schwahn et al. 2014). SGAs, commonly found in *Solanum* species, exhibit various biological activities, including

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toxicity, that contribute to plant defense against pathogens and herbivores (Milner et al. 2011). α-Tomatine, the major SGA in tomato, is a well-known bitter-tasting compound that accumulates at high levels in all the plant tissues, especially in the leaves and immature green fruits (Rick et al. 1994, Friedman 2002). However, α-tomatine in immature fruit is metabolized to esculeoside A through neorickiioside B, lycoperoside C and prosapogenin A as candidate intermediates during fruit ripening. Esculeoside A is a predominant SGA in ripe fruit (Nohara et al. 2010, Iijima et al. 2013), and the metabolic conversion is hypothesized to proceed *via* C-23 hydroxylation and isomerization of α-tomatine, 23-*O*-acetylation of neorickiioside B, C-27 hydroxylation of lycoperoside C and 27-*O*-glucosylation of prosapogenin A to form esculeoside A (**Fig. 1**). Sl23DOX (identical to GAME31) of the DOX superfamily was recently found to catalyze C-23 hydroxylation of α-tomatine, and the reaction product was spontaneously further isomerized to neorickiioside B (Cárdenas et al. 2019, Nakayasu et al. 2020). In addition, GAME5 has been reported to catalyze 27-*O*-glucosylation of prosapogenin A to form esculeoside A, although the down-regulation of *GAME5* by virus-induced gene silencing did not result in esculeoside A reduction in red ripe fruit (Szymański et al. 2020). However, the genes involved in the metabolic conversion from neorickiioside B to prosapogenin A remain to be elucidated.

The later steps in the esculeoside A production metabolic pathway are probably correlated with ethylene biosynthesis and response (Iijima et al. 2009). Here, we hypothesized that E8, a biochemically uncharacterized DOX, is involved in the metabolic conversion. Our in vitro and in planta analyses demonstrated that E8, designated as Sl27DOX, catalyzed C-27 hydroxylation of lycoperoside C to produce prosapogenin A. These results provide a better understanding of the metabolic changes in SGAs during tomato fruit ripening and their variations in domesticated and wild tomato fruit.

## Results

### C-27 hydroxylase candidate gene selection

In the metabolic pathway from α-tomatine to esculeoside A during tomato fruit ripening, lycoperoside C is thought to be hydroxylated at C-27 to form prosapogenin A (**Fig. 1**). We have previously screened *DOX* superfamily genes *in silico* to find candidates for α-tomatine metabolism from 239 *DOX* transcripts in the tomato genome and identified Sl23DOX as α-tomatine 23-hydroxylase (Nakayasu et al. 2020). Among the selected candidate genes, *Solyc03g095900* and *Solyc09g089580* were expressed at higher levels in ripening fruit than in mature green fruit, but their recombinant proteins did not metabolize α-tomatine (Nakayasu et al. 2020). In this study, we investigated whether *Solyc03g095900* and *Solyc09g089580* are responsible for C-27 hydroxylation of lycoperoside C. The *Solyc09g089580* gene is identical to *E8* that has been described in the database as a 1-aminocyclopropane-1-carboxylate oxidase (ACO) homolog.

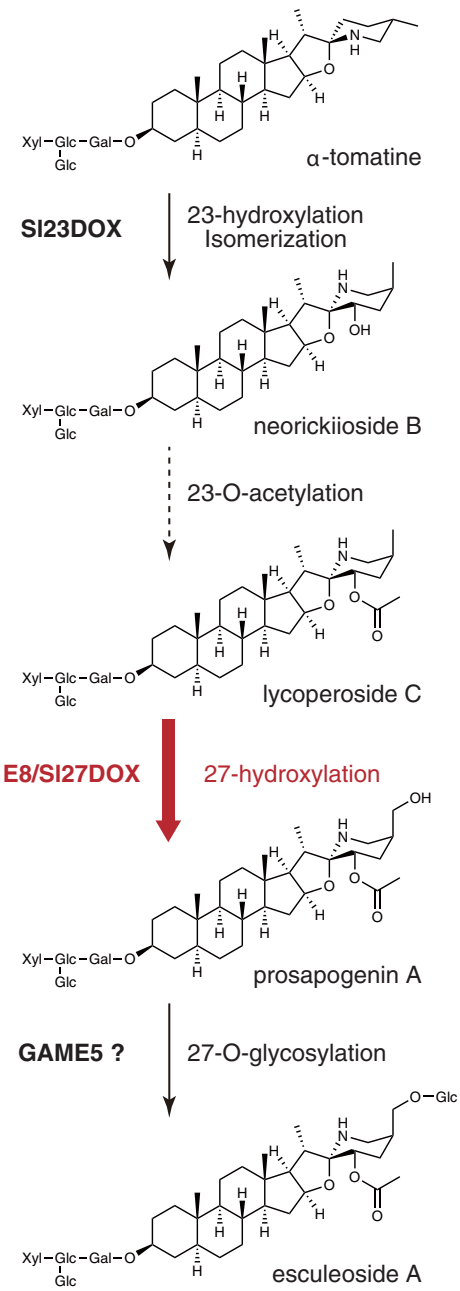


Fig. 1 Putative metabolic pathway from α-tomatine to esculeoside A during tomato fruit ripening. Black arrows represent characterized reactions. The dashed arrow indicates an unclear reaction stage. The reaction step characterized by E8/Sl27DOX is indicated using a red arrow.

However, E8 shares <40% amino acid sequence identity with ACOs, which belong to the DOXC53 clade (Kawai et al. 2014); therefore, E8's function seems to be distinctly different from the ACOs. Solyc03g095900 is described as an E8 protein homolog by the Solanaceae Genomics Network (https://solgenomics.net/). E8 and Solyc03g095900 share 75% amino acid sequence identity (**Supplementary Fig. S1**) and are classified in the DOXC31 clade that is phylogenetically different from the DOXC53 clade.

These proteins contain the Fe(II)-binding motif His-*X*-Asp-$X_n$-His (His-236, -244, Asp-238, -246 and His-292, -300 in E8 and Solyc03g095900) that is conserved in the DOX superfamily (Lukacin and Britsch 1997, Wilmouth et al. 2002, Bugg 2003). The Arg-*X*-Ser motif (Arg-302, -310 and Ser-304, -312 in E8 and Solyc03g095900) bind to the C-5 2-oxoglutarate carboxy group and are conserved in these proteins (Lukacin and Britsch 1997, Wilmouth et al. 2002).

## Catalytic function of *E8*-encoded recombinant enzyme

The glutathione S-transferase (GST) tagged recombinant proteins were prepared independently by a heterologous expression system in *Escherichia coli* to evaluate the enzymatic activity of E8 and Solyc03g095900. The recombinant E8 protein was expressed as a soluble protein, but Solyc03g095900 protein was expressed in an insoluble form; therefore, using a glutathione affinity column, we purified only the E8 recombinant protein (**Supplementary Fig. S2**). The catalytic activity of the recombinant E8 protein was assessed using lycoperoside C as a substrate. After incubation at 30°C for 1 h, the reactions were quenched by boiling and analyzed using liquid chromatography-mass spectrometry (LC-MS). This analysis showed that E8 metabolized lycoperoside C to a product with a retention time of 13.3 min (**Fig. 2A**). The product was identical to the authentic prosapogenin A, both in terms of its retention time and mass spectra (**Fig. 2**), confirming that E8 catalyzed C-27 hydroxylation of lycoperoside C to yield prosapogenin A. Hence, E8 was designated as Sl27DOX. Next, the chaperon proteins GroEL and GroES were co-expressed to prepare the Solyc03g095900 recombinant protein as a soluble protein. The expression of Solyc03g095900 in the soluble fractions was detected using SDS-PAGE (**Supplementary Fig. S2**). The soluble fraction containing the recombinant Solyc03g095900 was incubated with lycoperoside C; however, no new product was detected, suggesting that the catalytic function of Solyc03g095900 is different from E8/Sl27DOX (**Supplementary Fig. S3**).

## E8/Sl27DOX substrate specificity

The substrate specificity of E8/Sl27DOX toward various SGAs was examined (**Supplementary Fig. S4**). The purified recombinant E8/Sl27DOX did not metabolize α-solanine, α-chaconine, α-solamarine, β-solamarine or solasonine. Furthermore, α-tomatine and neorickiioside B, which are direct precursors of the substrate lycoperoside C, were not hydroxylated by E8/Sl27DOX at C-27, indicating the strict substrate specificity of E8/Sl27DOX and confirming the reaction order of the metabolic conversion from α-tomatine to esculeoside A (**Fig. 1**). The soluble fractions containing the Solyc03g09500 recombinant protein showed no activity on the above substrates (**Supplementary Fig. S4**). Next, the kinetic parameters of E8/Sl27DOX for lycoperoside C were investigated. The $K_m$ and $k_{cat}$ values were 30.1 μM and 2.55 $s^{-1}$, respectively

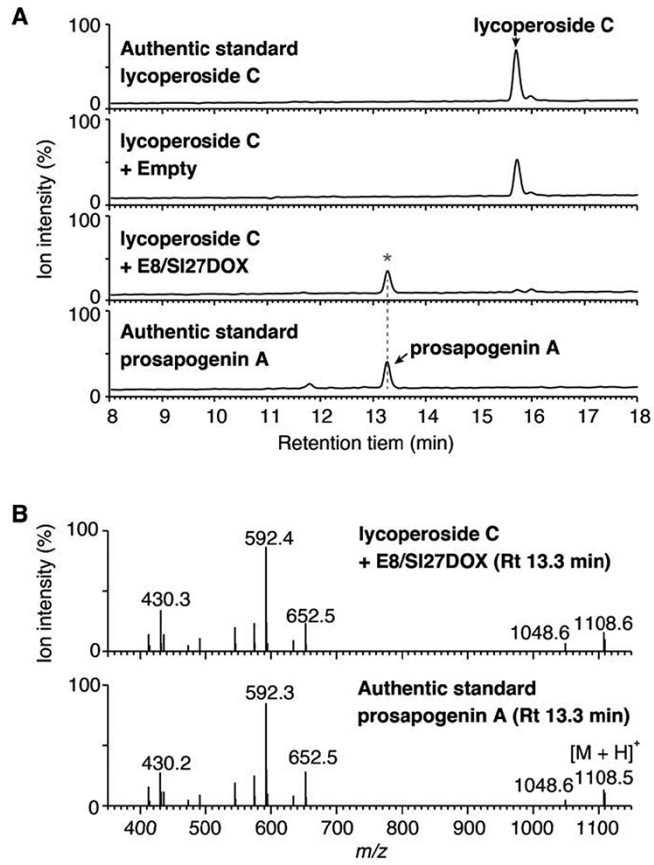


Fig. 2 LC-MS analysis of reaction products from the purified E8/Sl27DOX recombinant protein with lycoperoside C as a substrate. (A) Total ion chromatograms of reaction products and authentic compounds. (B) Mass spectra of peaks shown in A at 13.3 min retention time.

**Table 1** Kinetic parameters of the E8/Sl27DOX recombinant protein for lycoperoside C

| Parameter | Value |
|---|---|
| $K_m$ (μM) | 30.1 ± 0.0838 |
| $K_{cat}$ ($s^{-1}$) | 2.55 ± 0.137 |
| $K_{cat}/K_m$ ($\mu M^{-1}$ $s^{-1}$) | 0.0847 ± 0.00455 |

Data are the means of three replicates ± standard deviation.

(**Table 1**, **Supplementary Fig. S5**). These values are comparable with *Arabidopsis* feruloyl-CoA 6′-hydroxylase ($K_m$: 36.0 μM and $k_{cat}$: 11 $sec^{-1}$), a dioxygenase involved in coumarin biosynthesis (Kai et al. 2008).

## Characterization of *E8/Sl27DOX*-silenced transgenic tomato plants

*E8/Sl27DOX*-silenced transgenic tomato plants were constructed to confirm the contribution of *E8/Sl27DOX* to α-tomatine metabolism during tomato fruit ripening. The *E8/Sl27DOX* expression levels were analyzed by quantitative

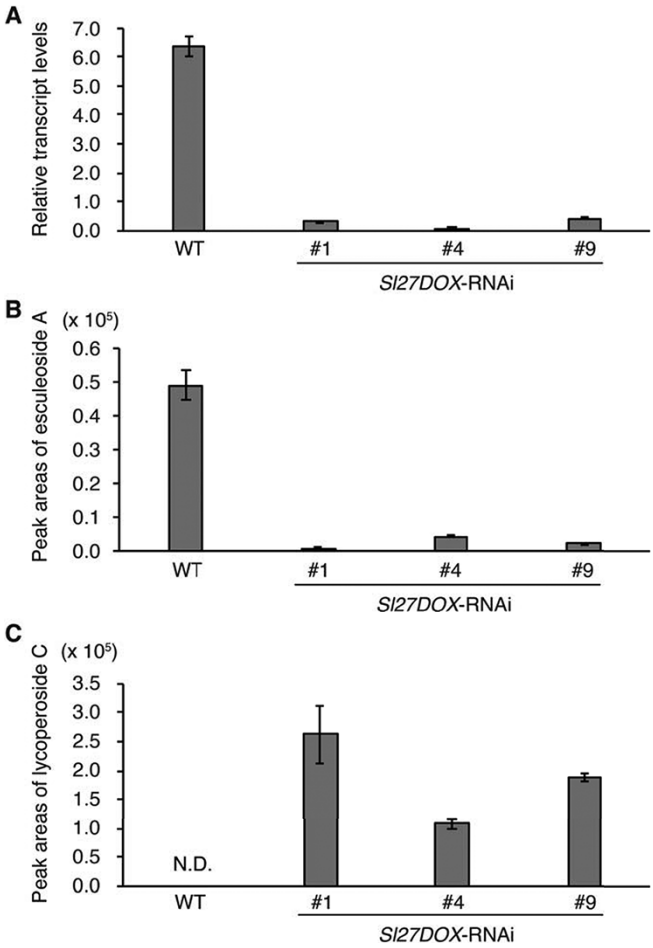


Fig. 3 Analysis of *E8/Sl27DOX*-silenced transgenic tomato plants. (A) Quantitative RT-PCR analysis of *E8/Sl27DOX* in red *E8/Sl27DOX*-silenced transgenic tomato fruit. (B) Analysis of esucleoside A accumulation levels in red *E8/Sl27DOX*-silenced transgenic tomato fruit. (C) Analysis of lecoperoside C accumulation levels in red *E8/Sl27DOX*-silenced transgenic tomato fruit. Error bars indicate standard deviation ($n = 3$). WT, wild-type plant (control); #1, #4, and #9: independent transgenic lines.

RT-PCR using the total RNA extracted from fruits harvested 20 days after color turning. The results revealed that the three independent lines (#1, #4, and #9) exhibited ≥90% reduction in *E8/Sl27DOX* expression level compared with the wild-type (WT) plants (**Fig. 3A**). The SGA profile analysis of fruit at the same growth stage used for expression analysis revealed that the *E8/Sl27DOX*-silenced lines exhibited ≥90% esculeoside A reduction than WT (**Fig. 3B**). Furthermore, the *E8/Sl27DOX*-silenced lines were associated with lycoperoside C accumulation, consistent with a decrease in esculeoside A (**Fig. 3C**). These results demonstrated that *E8/Sl27DOX* is a key gene in C-27 hydroxylation of lycoperoside C in the metabolic conversion of α-tomatine to esculeoside A during tomato fruit ripening.

## *E8/Sl27DOX*-deficient tomato accessions

Zhu et al. (2018) reported a dataset encompassing genomes, transcriptomes and metabolomes from hundreds of tomato genotypes. We found three accessions (TS-609: heirloom tomato, cv. Pineapple, TS-106: *S. lycopersicum* var *cerasiforme*, unknown cultivar from Costa Rica, and TS-53: *S. lycopersicum* var *cerasiforme*, LA2095) that showed glycoalkaloid-metabolite fruit-pericarp profiles similar to the *E8/Sl27DOX*-silenced transgenic tomatoes. These accessions were higher in lycoperoside C and lower in esculeoside A than the representative tomato strains with normal metabolism from α-tomatine to esculeoside A (Micro-Tom, Alisa Craig, Heinz) (**Supplementary Table S1**). Next, we analyzed *E8/Sl27DOX* and *Solyc03g095900* expression in their fruits using the fruit-pericarp transcriptome datasets (SRR5932894/TS-609, SRR5932955/TS-106, SRR5933090/TS-53, SRR5932973/Micro-Tom, SRR5932975/Ailsa Craig and SRR5933194/Heinz 1706-BG). *E8/Sl27DOX* expression in the three lycoperoside C accumulation strains (TS-609, TS-106 and TS-53) was significantly lower than the control tomato strains with normal α-tomatine metabolism (Micro-Tom, Alisa Craig, Heinz), although *Solyc03g095900* expression was similar among them (**Supplementary Table S2**). The short-read genome sequences of the three accessions and the control strains were mapped to the tomato genome ITAG4.0 (Solanaceae Genomics Network) and visualized using the Integrative Genomics Viewer: http://software.broadinstitute.org/software/igv/ (**Supplementary Fig. S6**). The short-read genome sequences of the control strains were mapped on *E8/Sl27DOX*; however, those of the three accessions were not (**Fig. 4**). These results suggested that almost the entire *E8/Sl27DOX* gene locus of the three accessions (TS-609, TS-106, and TS-53) was deleted, likely causing a loss of *E8/Sl27DOX*-encoded C-27 hydroxylase that could lead to lycoperoside C accumulation in the accessions. Alonge et al. (2020) reported that the surrounding region of *E8/Sl27DOX* gene is a region with genomic structure variations among diverse tomato lines. They identified the genotype having a large deletion that remove *E8/Sl27DOX* and its surrounding region, and also reported that TS-609, TS-106 and TS-53 are classified into the genotype. This is in line of our observations.

## Phylogenetic analysis and genomic organization of *E8/Sl27DOX* and homologs

Our in vitro and in vivo analyses demonstrated that E8/Sl27DOX is necessary for α-tomatine detoxification during fruit ripening in cultivated tomatoes. The *E8/Sl27DOX* gene is located on chromosome 9 in the tomato genome, and we found that it exists as part of a gene cluster, comprising highly tandemly duplicated DOX superfamily genes (**Supplementary Data 1**). Moreover, *Solanum* species such as wild tomato relatives, potato and eggplant exhibited similar clustering of highly duplicated DOX superfamily genes at the corresponding region on chromosome 9 (**Supplementary Data 1**). Next, we investigated whether *E8/Sl27DOX* orthologous genes are present in other SGA-producing *Solanum* species. The investigation revealed that *S. pimpinellifolium*, the direct ancestor of domesticated tomatoes, possesses an E8/Sl27DOX ortholog exhibiting 100% amino acid sequence identity with E8/Sl27DOX. Likewise, we confirmed the presence

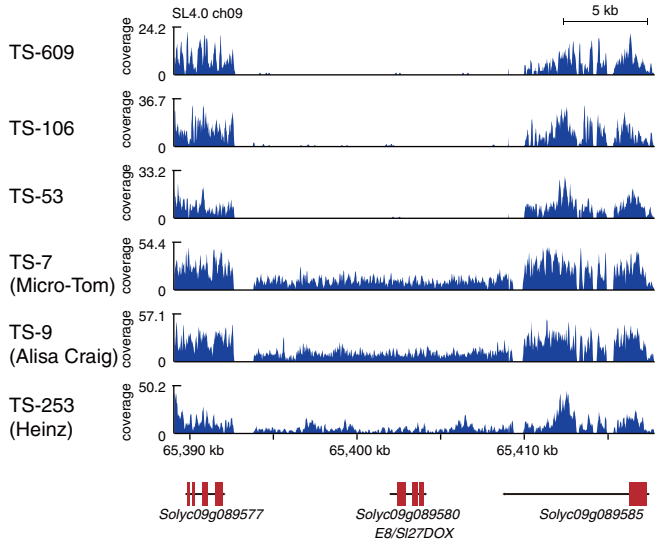


Fig. 4 Coverage track of short-read genome sequences mapped at *Solyc09g089580* (*E8/Sl27DOX*) and surrounding region. The binary alignment map coverage was visualized using SparK.

of an E8/Sl27DOX ortholog, with 98% amino acid sequence identity in another wild tomato relative, *S. pennellii*. In contrast, no E8/Sl27DOX orthologs were found in potato (*S. tuberosum*) or eggplant (*S. melongena*). Soltu.DM.03G013240.1 exhibits the highest amino acid sequence identity (76.8%) with E8/Sl27DOX among all potato DOXs and shares 90.0% amino acid sequence identity with Solyc03g095900, whose catalytic function is different from E8/Sl27DOX, as shown in this study. SMEL_009g331920.1.0 exhibits the highest amino acid identity (75.9%) with E8/Sl27DOX among the DOXs from eggplant and shares 77.2% amino acid identity with Solyc03g095900. To investigate the evolutionary origin of E8/Sl27DOX, we constructed the phylogenetic tree of DOX superfamily enzymes that shows more than 70% amino acid sequence identity to E8/Sl27DOX among five *Solanum* species (*S. lycopersicum, S. pimpinellifolium, S. pennellii, S. tuberosum*, and *S. melongena*) (**Fig. 5**). Tomato E8/Sl27DOX forms a phylogenetic cluster with E8/Sl27DOX orthologs from *S. pimpinellifolium* and *S. pennellii* that belong to a subclade containing no enzymes from potato or eggplant (**Fig. 5**). The presence of the E8/Sl27DOX orthologs, restricted in cultivated and wild tomato species, suggested that they emerged within the tomato lineage, including wild tomato relatives, after the split from other *Solanum* species.

## Discussion

In this study, we selected *E8* as a candidate gene involved in the metabolic conversion of α-tomatine during fruit ripening, and the in vitro enzyme assays revealed that E8, which is designated as Sl27DOX, catalyzes C-27 hydroxylation of lycoperoside C to prosapogenin A. The silencing of the *E8/Sl27DOX* gene in transgenic tomato plants demonstrated that E8/Sl27DOX primarily contributes to C-27 hydroxylation in the conversion during fruit ripening. Furthermore, the tomato accessions that contain high levels of lycoperoside C in fully ripe fruit displayed *E8/Sl27DOX* gene deletion. These findings verify the function of E8 as a C-27 hydroxylase in α-tomatine metabolism.

Tomato originates from South America, and domesticated tomatoes have been developed from the wild tomato *S. pimpinellifolium* as the ancestor species *via* domesticated cherry tomato (*S. lycopersicum* var. *cerasiforme*) (Lin et al. 2014). Iijima et al. (2013) performed SGA profiling of domesticated and wild tomato fruit. *S. pimpinellifolium* (LA1589), which produces small and red fruits on ripening, contains a low level of α-tomatine and a high level of esculeoside A, and its SGA profile is similar to domesticated tomatoes. In contrast, other wild tomatoes such as *S. pennellii* (LA0716) and *S. habrochaites* (LA1777), which produce green fruit even after ripening, contain a low level of esculeoside A and primarily accumulate α-tomatine and lycoperoside C (Iijima et al. 2013). The *S. pennellii* genome contains an *E8/Sl27DOX* orthologous gene. Thus, the regulation of the *E8/Sl27DOX* expression is likely a key factor in the metabolic conversion of α-tomatine. Previous studies have reported that *E8/Sl27DOX* orthologous gene transcription levels during fruit repining in *S. pennellii* are lower than in *S. lycopersicum* (Bolger et al. 2014). The difference in the *E8/Sl27DOX* promoter sequences between domesticated and wild tomatoes might affect the ethylene-responsive expression *E8/Sl27DOX*, leading to the highly efficient conversion of α-tomatine into esculeoside A in domesticated tomatoes due to the strong induction of *E8/Sl27DOX* during fruit ripening. α-Tomatine confers bitterness and antinutritional properties; therefore, humans have selectively bred tomatoes to reduce the fruit α-tomatine content (Rick et al. 1994). The domestication from *S. pimpinellifolium* is reasonable from the viewpoint of the SGA profile of ripe fruit. The domestication process changed the GAME9 transcription factor allele frequency for SGA biosynthesis regulation, resulting in a lower SGA content in domesticated tomato than in *S. pimpinellifolium* (Zhu et al. 2018).

Ethylene is the phytohormone responsible for tomato fruit ripening (Alexander and Grierson 2002). The metabolic conversion of α-tomatine to esculeoside A during tomato fruit ripening is also associated with ethylene signaling, and exogenous ethylene treatments enhanced the metabolic conversion (Iijima et al. 2009). Furthermore, tomato ripening mutants such as *ripening inhibitor* (*rin*, LA3012), *non-ripening* (*nor*, LA3013) and *Never-ripe* (*Nr*, LA3001) accumulated lower levels of esculeoside A and higher levels of its biosynthetic intermediates than the non-mutant tomatoes (Iijima et al. 2009). *E8/Sl27DOX*, transcriptionally upregulated by ethylene, is transcribed at lower levels in the ripening mutants than in non-mutant tomato (Yen et al. 1995). The MADS-box transcription factor, RIN, encoded by the *rin* locus, directly targets many genes, including *E8/Sl27DOX* and regulates various ripening processes (Fujisawa et al. 2013). Moreover, it has been reported that *E8/Sl27DOX* is regulated by several other transcription factors, including FUL1/2 and SlEFR.E2/4 (Fujisawa et al. 2014, Liu et al. 2016,

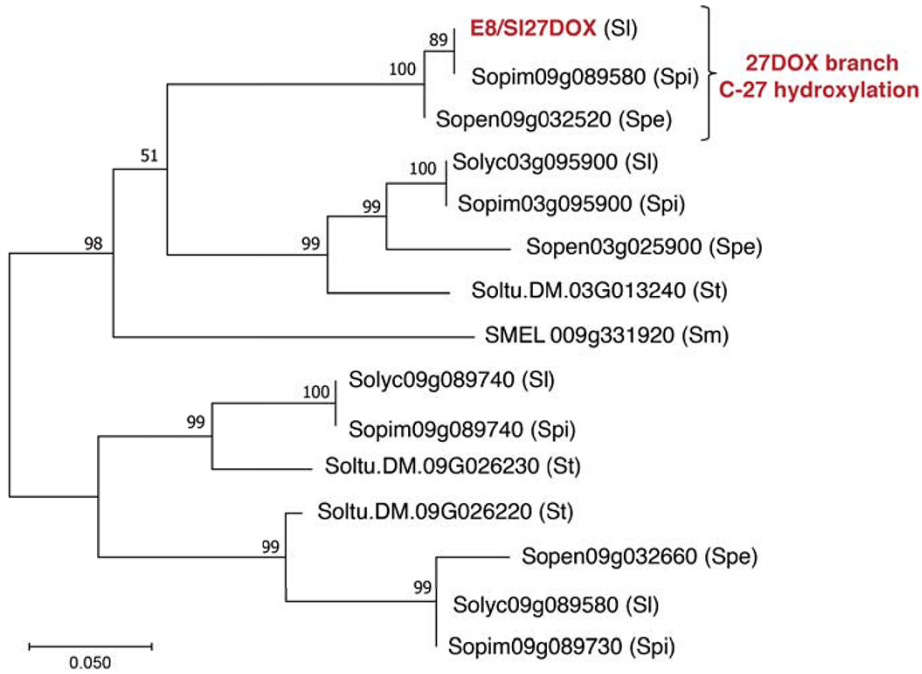


Fig. 5 E8/Sl27DOX maximum likelihood phylogenetic tree showing most closely related proteins. Bootstrap values based on 1,000 replicates are shown at the branching points. Sl, *S. lycopersicum* (tomato); Spi, *S. pimpinellifolium*; Spe, *S. pennellii*; St, *S. tuberosum* (potato); Sm, *S. melongena* (eggplant).

Wang et al. 2016). These findings suggest that the expression of genes involved in SGA metabolic conversion is highly regulated by ethylene-associated genes during fruit ripening.

DOX is the second-largest enzyme superfamily in plant genomes and is phylogenetically classified into the three classes, DOXA, DOXB and DOXC, which are subdivided into 57 DOXC clades (Kawai et al. 2014). Sl16DOX/GAME11 in SGA biosynthesis belongs to the DOXC41 clade (Itkin et al. 2013, Nakayasu et al. 2017), whereas Sl23DOX/GAME31 belongs to the DOXC20 clade (Cárdenas et al. 2019, Nakayasu et al. 2020). Furthermore, potato DPS (dioxygenase for potato solanidane synthesis) involved in the ring arrangement from spirosolanes to solanidanes in potato α-solanine biosynthesis belongs to the DOXC20 clade (Akiyama et al. 2021). In contrast, E8/Sl27DOX belongs to the DOXC31 clade, which is phylogenetically distinct from DOXC20 and DOXC41. DOXC31 includes GSL-OH, ZmBX6 and CrD4H, which are involved in the biosynthesis of glucosinolate in *Arabidopsis thaliana*, benzoxazinoid in *Zea mays*, and monoterpenoid indole alkaloid in *Catharanthus roseus* (Vazquez-Flota et al. 1997, Frey et al. 2003, Hansen et al. 2008), suggesting that DOXC31 contains functionally diverse DOXs responsible for lineage-specific metabolites (Kawai et al. 2014). *DOXC31* is the largest clade, which consists of 39 DOXC31 members among 239 *DOXs*, including pseudogenes in the domesticated tomato genome. Among them, 16 genes, including E8/Sl27DOX, are organized as *DOXC31* tandem repeats on chromosome 9 that are also observed in other Solanaceae plant genomes (**Supplementary Data 1**). Several examples have shown that tandem repeat regions are involved in the biosynthesis of specialized plant metabolites such as glucosinolate, flavonoids, and terpenoids and tandem gene duplication can result in neofunctionalization to produce metabolic diversification (Peng et al. 2017, Cárdenas et al. 2019). Therefore, a comprehensive functional analysis of DOXC31 members in the Solanaceae genome tandem repeat regions could provide insights into the evolutionary history of E8/Sl27DOX acquisition and SGA diversity in Solanaceae.

## Materials and Methods

### Chemicals

An authentic sample of α-tomatine was purchased from the Tokyo Chemical Industry (Tokyo, Japan). Neorickiioside B, lycoperoside C, prosapogenin A and esculeoside A were isolated as previously described (Iijima et al. 2013). α-solanine, α-chaconine and solasonine were purchased from Sigma-Aldrich (St. Louis, MO, USA), Extrasynthese (Genay, France) and AvaChem Scientific (San Antonio, TX, USA), respectively. α-solamarine and β-solamarine were isolated in previous study (Shimizu et al. 2020).

### Recombinant protein expression

*E8/Sl27DOX* DNA fragments and Solyc03g095900 coding sequences had been inserted into pGEX4T-1 (Takara Bio, Shiga, Japan) in a previous study (Nakayasu et al. 2020). The *E. coli* strain BL21 (DE3) (Clontech, CA, USA), transformed with a constructed plasmid, was incubated at 37°C in a lysogeny broth medium containing ampicillin (50 μg $ml^{-1}$) until the OD600 reached 0.5. Recombinant protein production was induced by adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the culture was incubated for 24 h at 18°C, followed by centrifugation at 8,000 rpm for 10 min at 4°C. The cell pellets

were resuspended in 5 ml cold sonication buffer containing 50 mM bis-Tris–HCl (pH 7.2), 150 mM NaCl, 10% (v/v) glycerol and 5 mM dithiothreitol. Then, the solution was sonicated on ice thrice for 30 s each, using an MS73 Bandelin Sonopuls HD 2070 ultrasonic homogenizer (Sigma-Aldrich) at 200 W $cm^{-2}$ sound intensity and centrifuged at 15,000 rpm for 10 min at 4°C. The GST-tagged Sl27DOX recombinant protein purification was conducted as previously described (Nakayasu et al. 2017). In co-expression with chaperon proteins, the constructed vector was introduced into the *E. coli* strain BL21 (DE3) containing a chaperon plasmid, pGro12 (Nishihara et al. 1998). The transformed *E. coli* was incubated at 37°C in lysogeny broth that included 50 μg $ml^{-1}$ ampicillin, 50 μg $ml^{-1}$ kanamycin and 0.5 mg $ml^{-1}$ L-arabinose until the OD600 reached 0.5. Recombinant protein expression was induced by adding 0.1 mM IPTG and maintained for 24 h at 18°C.

## Recombinant enzyme assays

In the *in vitro* assay, the reaction mixture (100 μl) consisted of 100 mM bis-Tris–HCl (pH 7.2), 5 mM 2-ketoglutaric acid, 10 mM sodium ascorbate, 200 μM $FeSO_4$ and 100 μM lycoperoside C as a substrate, and the purified E8/Sl27DOX recombinant protein. The enzymatic reaction was conducted at 30°C for 60 min and stopped by heat treatment at 100°C. The mixture was centrifuged at 15,000 rpm for 10 min, and the supernatant was diluted with methanol and filtered using a 0.2 μm polytetrafluoroethylene filter (Waters, Milford, MA, USA). LC-MS analysis of the reaction products was performed as previously described (Abdelkareem et al. 2017) with the following minor modifications: water with 0.1% (v/v) formic acid (A) and acetonitrile (B); gradient conditions of solvent B ramped linearly from 10% to 40% over 20 min; solvent B increased linearly to 100% over 2 min, held at solvent B 100% for 3 min; solvent B immediately returned to 10%, followed by 5 min re-equilibration; sample cone voltage at 80 V and mass spectra obtained by the MS scan mode with a mass range of *m*/*z* 350–1,250.

## Kinetic analysis of E8/Sl27DOX recombinant protein

We determined the kinetic parameters of the purified E8/Sl27DOX recombinant protein in triplicate assays. The reaction mixture was prepared as described above with modifications: the volume was 200 μl, sodium phosphate (pH 7.0) was used instead of bis-tris–HCl (pH 7.2) and concentration of lycoperoside C ranged from 10 to 100 μM. The reaction was carried out at 35°C for 15 min. The reaction products were extracted thrice, with an equal volume of *n*-butanol containing genistin (1 μg $ml^{-1}$) as an internal standard. The collected organic phase was evaporated and dissolved in 50 μl methanol, followed by sonication for 30 min. LC-MS analysis was conducted as described above with modifications: the sample injection volume was 5 μl, UV absorbance at 254 nm was monitored to quantify genistin, and selected ion recording mode with *m*/*z* 1,108.5 was applied to quantify prosapogenin A. The peak areas of the two compounds were divided by the area of genistin as the internal standard, and the values were used to calculate the contents of the two products using a prosapogenin A calibration curve. The kinetic parameters were determined by nonlinear regression using the ANEMONA program (Hernandez and Ruiz 1998).

## *Sl27DOX*-silenced transgenic tomato plant generation

The tomato, *S. lycopersicum* cv. Micro-Tom (TOMJPF00001), plants were transformed with RNAi constructs designed from the protein-coding region of the target gene to silence the transcript. Partial *E8/Sl27DOX* (250 bp) coding sequence DNA fragments were PCR-amplified using primer sets 5′-CATATGGAATTCGATTAAGCATAAGGAGAT-3′ and 5′-GTCGACGGATCCCG TGTCTCTCCAACTT-3′. An RNAi binary vector for *E8/Sl27DOX*-silencing was constructed using pRI 101-AN-DNA (TaKaRa) as the backbone by locating the two partially opposing *E8/Sl27DOX* fragments interposing the third intron of the *A. thaliana* gene *At4g14210* under the cauliflower mosaic virus 35S (CaMV35S) promoter. The constructed plasmid was electroporated into *Agrobacterium tumefaciens* C58 cells. Stable transgenic plants tomatoes were generated as previously described (Sun et al. 2006). The transformants were selected by genomic PCR of the leaves, using primers 5′-ATGATTGAACAAGATGGATTGC-3′ and 5′-TCAGAAGAACTCGTCAAGAAG-3′ targeting the kanamycin resistance gene.

## Quantitative RT-PCR analysis

Total RNA was prepared from red fruits sampled 20 days after color turning using the RNeasy plant mini-kit (QIAGEN, Hilden, Germany) and an RNase-Free DNase Set (QIAGEN). The extracted total RNA was used to synthesize the first-strand cDNA using a Transcriptor First Strand cDNA Synthesis Kit (TOYOBO, Osaka, Japan). Quantitative RT-PCR analysis of *E8/Sl27DOX* was conducted with LightCycler®Nano (Roche, Basel, Switzerland) with THUNDERBIRDTM SYBR® qPCR Mix (TOYOBO) using primer sets 5′-AACTCCATGCGGGGAGTCAT-3′ and 5′-GGTAGTAGTTGCAAGAACAGAAAAGATGAA-3′. Primers that targeted the Ubiquitin gene, 5′-CACCAAGCCAAAGAAGATCAAGC-3′ and 5′-TCAGCATAAGGGCACTCCTAACG-3′, were used to normalize the gene expression levels. Cycling was performed for 10 min at 95°C, 45 cycles of 10 s at 95°C, 10 s at 59°C and 15 s at 72°C for amplification, followed by holding for 30 s at 95°C and ramping up from 60 to 95°C at 0.1°C $s^{-1}$ for melting curve analysis. Three biological repeats were analyzed in duplicate. Data acquisition and analysis were performed using LightCycler®Nano software (Roche).

## *E8/Sl27DOX*-silenced transgenic tomato fruit SGA analysis

Red *E8/Sl27DOX*-silenced transgenic tomato fruits were harvested 20 days after color turning and ground using a mortar and pestle. The SGAs were extracted thrice from 100 mg of the red fruit powder with 300 μl of methanol containing genistin (1 μg $ml^{-1}$) as an internal standard. The extracts were centrifuged at 15,000 rpm for 5 min at 4°C. The supernatant was diluted with an equal volume of methanol, filtered using a 0.2 μm PTFE filter (Waters), and analyzed by LC-MS/MS. LC-MS/MS analysis was conducted on a system composed of an Acquity ultra-performance liquid chromatography system (UPLC) (Waters) and an Acquity quadrupole tandem mass spectrometer (Waters). The data acquisition and analysis were performed using MassLynx 4.1 software (Waters). The extracts (2 μl) were separated with an Acquity UPLC HSS T3 (1.8 μm, 2.1 × 100 mm) (Waters). The column temperature was set at 30°C and the flow rate at 0.2 ml·$min^{-1}$. The mobile phases were 20% methanol (A) and 100% methanol (B), using a gradient elution of 0% B at 0–3 min, 0–30% B at 3–5 min, 30–50% B at 5–10 min, and 50–100% B at 10–25 min. The SGAs were detected using the multiple reaction monitoring mode (MRM). The MRM transitions were set at *m*/*z* 1,092.4 > 255.4, >414.4, >576.4, >1,014.4, and >1,032.4 for lycoperoside C and *m*/*z* 1,270.6 > 163.2, >545.2, and >1,210.5 for esculeoside A. The cone voltages were set at 65 V for lycoperoside C and 80 V for esculeoside A, and the collision energy at 50 eV for both compounds.

## De novo transcriptome assembly, gene expression analysis and genome mapping

De novo transcriptome assembly was constructed using Trinity-r2012-10-05 (Hass et al. 2013). The reads were mapped to contigs using Bowtie version 0.12.8 (Langmead 2010), and the expression level (fragments per kilobase of exon per million mapped fragments) was calculated using eXpress v1.2.2 for gene expression analysis (Roberts et al. 2011). Map short reads of the genome sequences (TS-609: SRR5080038, TS-106: SRR1572362, TS-53: SRR1572326, TS-7: SRR1572458, TS-9: SRR1572460 and TS-253: SRR1572628) to the tomato reference genome were analyzed by TopHat2 (TopHat-2.0.6; Kim et al. 2013) for genome mapping. The data were analyzed on the Maser platform (Kinjo et al. 2018). The binary alignment map coverage was visualized using SparK (Kurtenbach and Harbour 2019).

## Phylogenetic analysis

E8/Sl27DOX and its homologous sequences that exhibited >70% amino acid sequence identity with E8/Sl27DOX were selected from the protein database of

*Solanum* plants using the BLASTP (Basic Local Alignment Search Tool) program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence alignments were performed using MUSCLE (multiple sequence comparison by long-expectation) (Edgar 2004), and the maximum likelihood tree was inferred in MEGA10 (Kumar et al. 2018) using 1,000 bootstrap replications.

## Supplementary Data

**Supplementary data** are available at PCP online.

## Data Availability

Nucleotide sequence data for E8/Sl27DOX (LC623722) and Solyc03g095900 (LC623723) are deposited in DDBJ (DNA data bank of Japan). All gene sequences are available at the SolGenomics database (https://solgenomics.net) and Spud DB (http://solanaceae.plantbiology.msu.edu/).

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## Disclosures

The authors have no conflicts of interest to declare.

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